## REMARKS

Claims 20-23 and 30-46 are currently pending in this application. Claims 32-34, 36, and 37 have been allowed. By way of this Reply, claims 20, 23, and 35 have been amended and claims 38-49 have been added.

**Claims 20-23, 30, 31, and 46**

Claims 20-23, 30, and 31 were rejected under 35 U.S.C. § 112, first paragraph, for failing to comply with the written description requirement. The Examiner asserted "there is no disclosure, either explicit or implicit, of a distinct family of covalent heterodimeric proteins known as a 'CD94/NKG2' family." Independent claim 20 has been amended in accordance with the Examiner's remarks to specifically claim the relevant group of CD94/NKG2 receptors. It is noted that any alternative spliced form of the claimed group of NKG2 receptors would be included within this group as disclosed in the specification at pg. 3, ln. 27 - pg. 4, ln.5. Similarly, by way of background, NKG2H represents an alternative spliced form of the NKG2E gene. See Bellon et al., Triggering of Effector Functions on a $CD8^+$ T Cell Clone Upon the Aggregation of an Activatory CD94/kp39 Heterodimer, *The Journal of Immunology*, 162: 3996-4002 (1999), copy enclosed.

Accordingly, claims 20-23, 30, 31, and 46 should be in condition for allowance.

**Claims 23, 35, and 38-46**

Claims 23 and 35 were rejected under 35 U.S.C. § 102(b) as being anticipated by Aldrich et al. as evidenced by Brooks et al. The Examiner asserted that "the binding of HLA-E to CD94/NKG2 receptors is an inherent property of the AMAPRTLL peptide taught by Aldrich as evidenced by the post-filing date teachings of Brooks." However, Aldrich does not disclose compounds affecting the binding of HLA-E to CD94/NKG2 receptors as used in medical diagnostic procedures as recited in amended claims 23 and 35 and new claims 38 and 42.

Claims 23 and 35 were also rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement and under 35 U.S.C. § 112, first paragraph, for lack of enablement. These rejections will be addressed together because the Examiner asserted, with respect to both rejections, that claims 23 and 35 are broadly drawn to encompass the entire genus of "compounds" that affect the binding of HLA-E to CD94/CKG2 while the specification only teaches a small group of compounds having such properties. Independent claims 23 and 35 have been amended to recite that the compounds comprise small peptides as supported by the disclosure in the specification at the paragraph at pg. 11, ln. 23 – pg. 12, ln. 4, Table 1 on pg. 19, and Table 2 on pg. 26. Similarly, new claims 38-45 all recite that the compounds comprise antibodies as supported by the disclosure in

the specification at pg. 8, lns 3-17, and Examples 2, 3, 4, and 8 (spanning from pgs. 21- 36).

Accordingly, claims 23, 35, and 38-45 should be in condition for allowance.

**Claims 46-49**

Independent claim 46 has been added to recite a method for identifying compounds affecting the binding of HLA-E to specific CD94/NKG2 receptors. It is respectfully submitted that the method recited in claim 46 should be in condition for allowance.

Dependent claims 47-49 have been added to recite a use for the compounds determined by the claimed methods that affect the binding of HLA-E to the cells, which is not disclosed in the cited prior art. It is respectfully submitted that the methods recited in dependent claims 47-49 should be in condition for allowance.

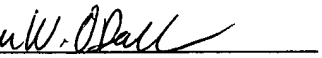
**Conclusion**

For the above reasons provided above, it is respectfully submitted that pending claims 20-23 and 30-49 are in condition for allowance. Accordingly, reconsideration and allowance of all pending claims is respectfully requested.

If the Examiner does not believe that the claims are in condition for allowance, the Examiner is respectfully requested to contact the undersigned at 215-568-6400.

Respectfully submitted,

Braud et al.

By 
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Enclosures

# Triggering of Effector Functions on a CD8$^+$ T Cell Clone Upon the Aggregation of an Activatory CD94/kp39 Heterodimer[1]

Teresa Bellón,[2]* Agustín B. de Heredia,[2†] Manuel Llano,* Alfredo Minguela[‡], Antonio Rodriguez,* Miguel López-Botet,* and Pedro Aparicio[3†]

**Some T lymphocytes express the CD94 Ag, which is known to form heterodimers with members of the NKG2 family. We have studied the expression pattern and function of CD94 heterodimers in different $\alpha\beta$ or $\gamma\delta$ T cell clones. Most of the CD94$^+$NKG2A$^-$ T cells have a low to intermediate expression of CD94 Ag. The cross-linking of the CD94/NKG2 heterodimer in one of these CD8 $\alpha\beta$ CD94$^+$NKG2A$^-$ T cell clones (K14B06) was able to: 1) increase the intracellular concentration of $Ca^{2+}$, 2) induce the up-regulation of CD25 Ag expression and the secretion of IFN-$\gamma$, and 3) trigger redirected cytotoxicity in a TCR-independent manner. This activatory property was not shared by any other costimulatory molecule expressed by the K14B06 T cell clone, including CD8, CD28, CD45, CD69, or CD2 Ags. The immunoprecipitation of CD94 heterodimer showed a 39-kDa band with a similar m.w. to the activatory heterodimer found on some NK clones. A novel form of the NKG2 family (NKG2H) was identified in K14B06. NKG2H protein represents an alternative spliced form of the NKG2E gene, displaying a charged residue in the transmembrane portion and a cytoplasmic tail that lacks immunoreceptor tyrosine-based inhibitory motifs. The expression of NKG2H in the cell membrane through its association to CD94 and DAP-12 molecules supports that it could form part of the activatory CD94/Kp39 heterodimer present on K14B06 cells.** *The Journal of Immunology,* **1999, 162: 3996–4002.**

Natural killer cell function results from a balance between signals given by inhibitory and activatory receptors (1–4). Two families of NK receptors for MHC class I molecules have been described on NK cells. One is composed of a set of membrane proteins of the Ig superfamily (Ig-SF)[4]. The aggregation of Ig-SF receptors with tyrosine-based inhibitory motifs (ITIMs) in the intracytoplasmic domains inhibits the lysis of NK susceptible target cells due to induced tyrosine phosphorylation and consequent recruitment of Src homology 2-containing phosphatases (5,6). In contrast, other Ig-SF receptors trigger NK cell-mediated lysis upon ligation with specific mAbs. These activatory receptors contain shorter intracytoplasmic domains lacking ITIMs and display a different transmembrane region that facilitates their interaction with the DAP-12 dimer, an immunoreceptor tyrosine-based activation motif-bearing 12-kDa protein similar to the CD3$\zeta$-chain (7). The second family of receptors for MHC class I molecules is composed of type II proteins with a C-type lectin domain. CD94 glycoprotein belongs to this group and is able to covalently assemble to distinct C-type lectins of the NKG2 family (8). Whereas the CD94/NKG2A heterodimer constitutes an inhibitory receptor (9, 10), the association of CD94 with the NKG2C protein, highly homologous to NKG2A but lacking ITIM motifs in the intracellular domain, displays a triggering role (11). Both CD94/NKG2A and CD94/NKG2C heterodimers recognize the nonclassical HLA-E class I molecule, although the HLA-E-bound peptides influence the fine recognition of the heterodimers (12–14).

A subset of activated T lymphocytes expresses NK class I-specific receptors that are capable of either inhibiting or costimulating T cell activation triggered via CD3/TCR complex (15–18). In contrast to NK cells, the engagement of Ig-SF NK-type activatory receptors present on T cells does not trigger redirected cytotoxicity, although it alters the strength of the T cell response at a given Ag dose (16–18).

In this paper, we have studied the functional consequences of the aggregation of CD94 heterodimers on T lymphocytes. We identified one $\alpha\beta$ CD8$^+$ T cell clone (TCC; K14B06) that expresses an activatory CD94 heterodimer that triggers redirected lysis in a TCR-independent manner. In addition, the cross-linking of this activatory receptor with anti-CD94 mAb increased the intracellular concentration of $Ca^{2+}$ and induced the up-regulation of CD25 Ag expression, as well as the secretion of IFN-$\gamma$. The immunoprecipitation of the heterodimer showed that CD94 was covalently linked to a 39-kDa glycoprotein. The specificity of this activating CD94-heterodimer does not seem to coincide with that of CD94/NKG2C present on NK cells since RMA-S cells stably transfected with HLA-E, which was stabilized in the cell surface with an appropriate nonapeptide, were not killed by K14B06 TCC. The identification of a novel NKG2 molecule, named NKG2H, that is transported to the membrane together with CD94 and DAP-12 suggests that the CD94/NKG2H heterodimer could be an

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[4] Abbreviations used in this paper: Ig-SF, Ig superfamily; TCC, T cell clone; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer inhibitory receptor; GFP, green fluorescent protein.



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activatory receptor present on the surface of K14B06 with a still unidentified ligand specificity.

## Materials and Methods

### *Reagents, cytokines, and mAbs*

Culture medium was RPMI 1640 (Life Technologies Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine (Life Technologies), 10% FCS (Life Technologies), and antibiotics. Recombinant IL-2 (gift of Hoffmann-La Roche, Nutley, NJ) was used in a range between 5 and 25 U/ml. The following murine mAb to human Ags were used: anti-human IL-2R (anti-CD25) from Becton Dickinson (Berenbodegem, Belgium); T3b (19) (anti-CD3), TS2/18 (anti-CD2), TP1/40 (anti-CD11a), TP 1/36 (anti-CD43), HP2/9 (anti-CD44), HP2/19 (anti-CD50), D3/9 (anti-CD45), and TP 55.3 (anti-CD69) were a gift from Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain) (20, 21); 3B1 (anti-CD94) was previously described (22); Z199 was kindly provided by Dr. Moretta (Università di Brescia, Brescia, Italy) (23); HP-F1 (anti-ILT2) was previously described (24); 9.3 mAb (anti-CD28) was kindly donated by Bristol-Myers (Seattle, WA); 3A1 (anti-CD7) was obtained from the American Type Culture Collection (ATCC, Manassas, VA); IOL48 (anti-CD48) and IOL54 (anti-CD54) were from Immunotech (Luminy, France); and PE-conjugated anti-mouse F(ab')2 IgG was from Caltag (San Francisco, CA).

### *Cell cultures*

Human lymphoblastoid cell lines GUS, LG-2, and LG-15 (EBV-transformed normal human B cells), RMA-S cell line stably transfected with HLA-E (RMA-S/HLA-E) (generous gift from Dr. E. Weiss, Universitat Monehen, Munich, Germany), and P815 (murine cell lines) were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 1% penicillin-streptomycin. MLC and cloning were performed according to a method described elsewhere (20, 21). In brief, $10^6$ responder cells were stimulated with $5 \times 10^4$ irradiated (6000 rad) lymphoblastoid cell lines (LCL) and $10^6$ irradiated (4000 rad) spleen cells as feeder cells. Ten days after stimulation, responding cells were cloned by limited dilution. Cells were seeded at 5, 2.5, 1.2, 0.6, and 0.3 cells per well in round-bottomed or Terasaki microwells containing $2 \times 10^4$ irradiated LCL and $2 \times 10^4$ irradiated spleen cells in the presence of 100 U/ml of IL-2. Proliferating clones derived from wells seeded with a number of cells that resulted in ≤63% growing wells were further expanded by seeding $10^4$ cloned cells in round-bottom wells containing feeder cells and IL-2. KK2, H6.6, and K14B06 TCC were derived from PBMC; B107A and HP10.21 TCC were derived from spleen cells; and P72C, P96D, and 53B TCC were derived from fetal thymus. In some experiments, cells were used 2–4 wk from the last stimulation ($CD25^{low}$, quiescent cells) (20).

### *Immobilized or soluble Ab stimulation assay*

To immobilize stimulating Ab, 96-well flat-bottom plates were precoated with sheep anti-mouse IgG (7.5 μg/ml in 0.1 M Tris-HCl buffer (pH 8.2), 40 μl/well) (Sigma, St. Louis, MO) by overnight incubation at 4°C. Unbound anti-mouse Ab was removed by washing the plate three times with saline solution (SS). Afterward, the appropriate Ab was allowed to bind by adding 50 μl of Ab-containing culture supernatants or Ab solutions. Abs were incubated for 1 h at room temperature, and the plates were washed three times with SS. Once plates were coated with stimulating mAb, TCCs were added in medium containing IL-2 (10 U/ml) at $0.5–1 \times 10^6$ cells/well.

### *Immunofluorescence analysis*

TCC were analyzed by one- or two-color immunofluorescence after staining with appropriate mAbs. Cells were analyzed on a FACS cytofluorometer (Becton Dickinson) by using LYSIS II program (Becton Dickinson).

### *Cytotoxicity assays*

$^{51}Cr$-release cytolytic assays were performed in V-bottom microtiter plates as described (25). Briefly, $5 \times 10^3$ $^{51}Cr$-labeled target cells were incubated with varying amounts of effector cells for 4 h at 37°C. The percentage of specific release (*SR*) was calculated as follows: $SR = 100 \times [(E - C)/(T - C)]$, in which $E$ is cpm release by incubating targets with T cells, $C$ is cpm release from targets incubated with medium alone, and $T$ is the total cpm releasable from targets with 0.05 M HCl. Redirected lysis assays were performed as above, except that P815 cells were preincubated for 30 min at room temperature with particular mAbs.

### *Peptide binding to HLA-E*

Peptide loading of HLA-E molecules on RMA-S cells was conducted as described (14). Briefly, RMA-S/HLA-E cells were incubated for 18 h at 26°C in the presence of 100 μCi/ml $Na_2$ $^{51}CrO_4$, and 100 μM of synthetic peptides solubilized in DMSO. Samples were used as targets in cytotoxic assays (see above). In parallel, cells were washed, stained with HLA-E-specific (3D12) mAb, and analyzed by flow cytometry.

### *IL-4 and IFN-γ secretion*

IL-4 and IFN-γ were measured in culture supernatants by sandwich ELISA method following the manufacturer's instructions (PharMingen, San Diego, CA.), using recombinant human ILs as standards, purified anti-IL-4 (8D4-8) and anti-IFN-γ (N1B42) as coating mAbs, and biotinylated anti-IL-4 (MP4-25D2) and anti-IFN-γ (4S.B3) as detection mAbs. Briefly, serial cytokine standards dilution in 3% BSA-PBS and undiluted supernatants harvested from T cell culture after 24 h of stimulation were incubated overnight with constant shaking at 4°C on mAb-coated plates. A 45-min incubation period with biotinylated mAb (0.5 μg/ml) in 3% BSA-PBS and a subsequent 30-min incubation with avidin-peroxidase (Sigma) were used for detection at 405 nm, using 2,2'-azino-bis(ethylbenzthiazoline-6-sulfonic-acid) substrate (ABTS; Sigma). Statistical analysis was conducted using the Kruskal-Wallis test to evaluate the homogeneity of means between groups.

### *Measurement of the $(Ca^{2+})_i$*

$(Ca^{2+})_i$ was determined with the fluorescent $Ca^{2+}$ indicator Quin 2-AM (Calbiochem, La Jolla, CA). Briefly, $10^7$ T cells/ml were incubated for 45 min at 37°C in RPMI 1640 with 10% FCS and 25 μM Quin 2-AM. After this incubation, cells were washed twice and resuspended in RPMI 1640 ($5 \times 10^7$ cel/ml). T cells ($10^7$) were added to 1.3 ml of an electrolitic solution (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 25 mM glucose, 16 mM HEPES, and 6 mM Tris (pH 7.2)) and transferred to a fluorometer quartz cuvette, stirred, and heated at 37°C. Fluorescence was monitored in a Perkin-Elmer (Norwalk, CT) LS-5 Luminiscence spectrometer with an excitation wavelength of 339 nm and an emission wavelength of 492 nm. $(Ca^{2+})_i$ was determined as described (26), by using the formula $(Ca^{2+})_i$ nM = $115 [(F - F_{min})/(F_{max} - F)]$, where $F$ indicates the fluorescence at the unknown $(Ca^{2+})_i$, $F_{max}$ is the fluorescence after the Quin 2-AM is released by the addition of 0.05% Triton X-100, and $F_{min}$ is the remaining fluorescence after chelating $Ca^{2+}$ with 100 mM EGTA. Triggering of K14B06 TCC was achieved by adding 5 μg/ml of mAb, followed by the addition of 20 μg/ml polyclonal sheep anti-mouse IgG (Sigma).

### *Radiolabeling and immunoprecipitation*

Cells were surface-labeled with $^{125}I$ (Amersham, Buckinghamshire, U.K.) and lysed, and solubilized proteins were immunoprecipitated as described (9). Samples were analyzed by SDS-PAGE, followed by autoradiography.

### *RT-PCR*

Total RNA was extracted from K14B06 TCC by the acidic phenol method (27). Reverse transcription generating first strand cDNA was performed using 1 μg of total RNA and random hexamers in a total volume of 20 μl. First strand cDNA (5 μl) were used as template for PCR with specific primers. Primers used were: NKG2C (5'-ATGAGTAAACAAAGAG GAACCTTC-3'), which contains the ATG initiation codon; NKG2#3 (5'-CTCTAAAGCTTATGCTTACAATGAT-3'), which spans NKG2C termination codon; and NKG2X3'UT (5'-AAATAACACAATTCATTTTA AGGC-3'). PCR products were subcloned in pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. To construct the chimeric pEGFP-NKG2H vector, NKG2H cDNA was excised from pCR2.1 with *Bam*HI and *Eco*RV restriction enzymes and subcloned into *Bgl*II and *Sma*I sites of pEGFP-C1 polycloning site (Clontech, Palo Alto, CA). DAP-12 cDNA was PCR amplified from NK cells according to published sequence (7) and subcloned into *Xba*I and *Eco*RI sites of pJFE14 vector.

For fluorescence experiments COS-7 cells were seeded onto glass coverslips and transfected using the DEAE-Dextran method as described (9). After 48 h posttransfection, cells were fixed, incubated with specific mAb, washed, stained with a Cy-3-tagged goat anti-mouse IgG (Amersham), and fluorescence analyzed. Images were acquired with a COHU high performance CCD camera (Chou, Tokyo, Japan) coupled to the microscope and connected to a Leica (Cambridge, U.K.) Q550CW workstation. Images were visualized, processed, and stored by using Leica QFISH software version VI-01.

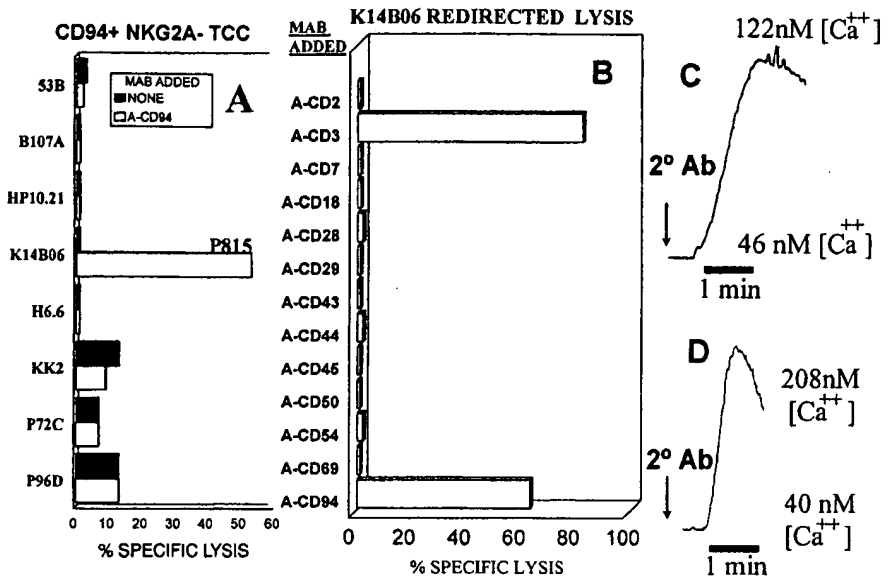



FIGURE 1. The addition of anti-CD94 mAb induced the lysis of P815 cell line in redirected cytotoxic assay and $(Ca^{2+})_i$ mobilization. *A*, Different $\alpha\beta$ $CD8^+CD94^+$ TCC (53B, B107A, HP10.21, and K14B06) and $\gamma\delta$ $CD94^+$ TCC (H6.6, KK2, P27C, and P96D) negative for the Z199 epitope were analyzed in a redirected killing assay against P815 target cells in the presence of 1.25 $\mu$g/ml of anti-CD94 mAb at a 2:1 E:T ratio. This experiment represents one of three with similar results. *B*, The $\alpha\beta$ $CD8^+$ TCC K14B06 was analyzed in a redirected killing assay against P815 target cells in the presence of several mAb specific for costimulatory T cell molecules at a 2:1 E:T ratio. The final concentration of anti-CD28 mAb was 1.25 $\mu$g/ml, whereas the other mAb were used as culture supernatants (1/4 final dilution). *C* and *D*, K14B06 TCC was analyzed for $(Ca^{2+})_i$ mAb mobilization after stimulation with anti-CD94 (*C*) or anti-CD3 (*D*) followed by goat anti-mouse IgG (2° Ab).

## Results

### *Consequences of CD94 aggregation in the cytotoxicity of the $CD94^+$ TCC*

Redirected lysis assays have been extensively used to show that the aggregation of the CD94/NKG2A heterodimer (recognized by the mAb Z199 (23)) inhibits the redirected cytotoxicity of NK clones, whereas the cross-linking of CD94 stimulates the lysis of target cells by $CD94^+Z199^-$ NK clones (28). We studied the functional consequences of these procedures on $CD94^+$ $\alpha\beta$ and $\gamma\delta$ TCC. In accordance with previous data (15, 29), the addition of anti-CD94 did not trigger redirected lysis in the absence of anti-CD3 or PHA on the TCC bearing the inhibitory CD94/NKG2A heterodimer ($CD94^+Z199^+$ TCC), although it was able to inhibit the redirected lysis of P815 in the presence of anti-CD3 or PHA in some of these clones (3 $\gamma\delta$ TCC of 15 $\alpha\beta$ and $\gamma\delta$ TCC tested (data not shown)). In contrast, one in eight $CD94^+Z199^-$ TCC tested (named K14B06) killed the P815 cell line in the presence of anti-CD94 mAb (Fig. 1*A*). The addition of anti-CD94 mAb did not modify the mitogen or Ab-redirected lysis of P815 by the other seven $CD94^+Z199^-$ TCC tested. It should be noted that the surface expression of CD94 on the K14B06 TCC was a stable phenotype during a prolonged culture period. To study whether the triggering of cytotoxicity on K14B06 TCC was restricted to the engagement of CD94 or if it was a property also shared by other membrane molecules, the redirected lysis of P815 by K14B06 was tested in the presence of different mAbs. As shown in Fig. 1*B*, none of the mAb tested, which were specific for a wide range of membrane molecules, was able to stimulate the activation of the TCC, suggesting that the signals induced by the aggregation of CD94 are different from those transduced by other costimulatory and/or accessory molecules.

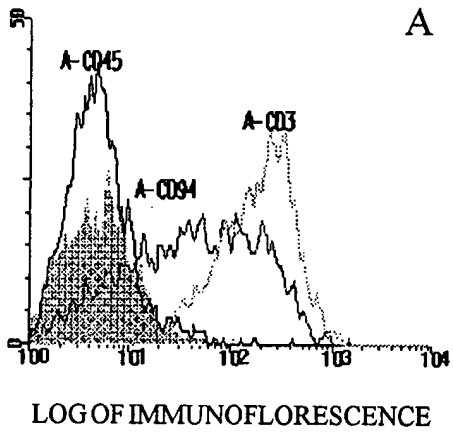
### *The aggregation of CD94 heterodimer in the absence of CD3 engagement triggers several activatory events on K14B06 TCC*

We tested whether the aggregation of CD94 on K14B06 increased the intracellular concentration of calcium, as shown in Fig. 1*C*. The addition of anti-mouse Abs to anti-CD94 preincubated K14B06 induced a 3-fold increase in intracellular calcium levels, attaining a calcium increase similar to that observed upon anti-CD3 cross-linking (Fig. 1*D*). The addition of anti-mouse Abs to either nontreated or to anti-CD2 preincubated K14B06 had no effect on the intracellular concentration of $Ca^{2+}$ (data not shown).

To clarify whether the aggregation of CD94 on the K14B06 TCC had other physiological effects besides the triggering of cytotoxicity, we added "quiescent" $CD25^{dim/-}$ K14B06 cells (see *Materials and Methods*) to wells with immobilized mAb. As shown in Fig. 2*A*, the aggregation of CD94 heterodimer, but not that of CD45 Ag, not only up-regulated the expression of CD25 (Fig. 2*A*) but also induced IFN-$\gamma$ secretion (Fig. 2*B*). Twenty-four-hour supernatants of anti-CD94-stimulated K14B06 TCC contained significantly more IFN-$\gamma$ (98.2 $\pm$ 39.1 pg/ml) than either nonstimulated cultures (25.1 $\pm$ 22.33 pg/ml, $p < 0.005$) or anti-CD45-stimulated cells (22.5 $\pm$ 9.8 pg/ml, $p < 0.001$), although the cytokine concentration was significantly lower than that observed upon optimal anti-CD3 activation (241.8 $\pm$ 30.7 pg/ml, $p < 0.001$). None of the stimuli tested were able to induce IL-4 secretion (data not shown). In contrast, none of the other mAbs tested in Fig. 1*B* (only the anti-CD45 mAb is represented here) were able to modify CD25 expression (Fig. 2*A*) or induce IFN-$\gamma$ production, although some of them transduced costimulatory signals. As shown in Fig. 2*A*, coimmobilized anti-CD45 increased the IFN-$\gamma$ production (425 $\pm$ 114.59 pg/ml; Fig. 2*B*) triggered by optimal concentration of plastic-bound anti-CD3 (241.8 $\pm$ 30.7 pg/ml, $p < 0.005$) and increased the CD25 up-regulation induced by suboptimal concentrations of anti-CD3 mAb (data not shown).

### *Molecular characterization of the CD94/NKG2 heterodimer expressed on K14B06 TCC*

It has been reported that the activatory CD94/NKG2C heterodimer expressed on NK cells recognizes HLA-E molecules loaded with nonapeptides derived from HLA class I signal sequences (12, 14). To test whether the activatory CD94 heterodimer expressed by K14B06 T cells was also able to recognize HLA-E molecules, we used the TAP-deficient mouse cell line RMA-S transfected with HLA-E cDNA (30). Culture of these cells in the presence of exogenous peptides derived from certain HLA-leader sequences is able to stabilize HLA-E on the cell surface (see *Materials and Methods*). Two NK clones, which have been previously described as expressing CD94/NKG2C activatory receptor and as recognizing HLA-E molecules loaded with peptides derived from HLA-G1



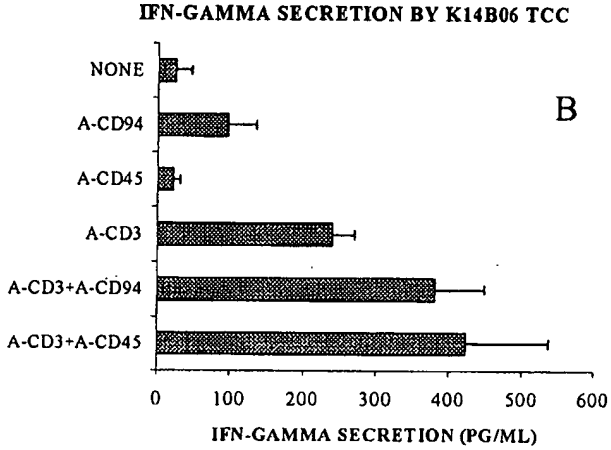




**FIGURE 2.** The aggregation of CD94 mediated CD25 up-regulation, and IFN-γ secretion by K14B06 TCC in the absence of CD3/TCR engagement. Sheep anti-mouse IgG $F(ab')_2$ precoated microtiter flat-bottom wells were allowed to bind to 2.5 μg/ml of either IgG1, T3b mAb (anti-CD3), RP2/21 (anti-CD45), or HP-3B1 (anti-CD94). K14B06 T cells ($1 \times 10^5$) were added to each coated microtiter well (0.2 ml/well). *A*, CD25 expression was analyzed 24 h later upon activation with these mAb: IgG1 (filled histogram), anti-CD45 (black open histograms), anti-CD94 (dark open histogram), and anti-CD3 (light open histograms). *B*, In parallel experiments, 24-h supernatants were collected and analyzed for the presence of IFN-γ. The data represented are the mean of three different experiments. The SD is represented as open rectangles.

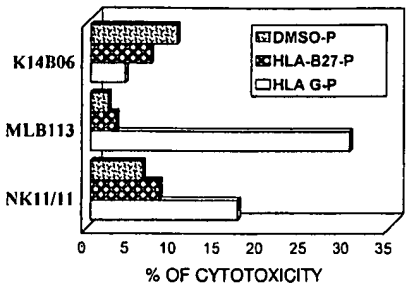



**FIGURE 3.** Cytotoxicity of K14B06 and other NK cell clones against peptide-loaded RMA-S/HLA-E cell line. K14B06 TCC and two CD94/NKG2C-expressing NK clones were tested in a $^{51}Cr$-release assay against HLA-E-transfected RMA-S cells loaded with synthetic peptides corresponding to B2705 (VTAPRTLLL) or HLA-G1 (VMAPRTLFL) signal sequences. This experiment represents one of three with similar results.

signal sequences (14), were compared with K14B06 TCC for their ability to recognize HLA-E on the surface of RMA-S cells in a $^{51}Cr$-release cytotoxic assay. As shown in Fig. 3, both NK cell clones were capable of killing cells cultured in the presence of a peptide that stabilizes HLA-E on the cell surface (HLA-G1), but not in the presence of B2705-derived peptide as previously described (14). In contrast, K14B06 TCC cytotoxic activity was not triggered under such experimental conditions.

We performed biochemical analysis of the activatory CD94 receptor present on K14B06 TCC. NKL cell line, which expresses NKG2A/CD94 inhibitory receptor, was included in the experiment for comparative purposes. As shown in Fig. 4*B*, the immunoprecipitation with anti-CD94 mAb resulted in the isolation of a 68-kDa complex in nonreducing conditions. Under reducing conditions (Fig. 4*A*) a single 39-kDa band is detected, similar to that described for the activatory form of CD94/NKG2 complex on NK cells (28). We studied the presence of mRNA for NKG2A, NKG2C, and NKG2E by reverse PCR in K14B06 TCC. Primers corresponding to NKG2C cDNA sequence amplified a specific band of the expected size from K14B06 TCC RNA. However, sequencing of this PCR product yielded a nucleotide sequence coding a NKG2 protein that also lacked ITIM motifs in the cytoplasmic tail, but that showed some nucleotide differences with NKG2C and did not contain a STOP codon. This new sequence was highly homologous to the sequence contained in a clone that had been previously isolated in the laboratory from an NK cDNA library that was lacking the 5′ sequences coding the NKG2 cytoplasmic region. Using a 5′ primer corresponding to NKG2C cDNA spanning the start codon sequence and a primer corresponding to the putative 3′ untranslated region sequence contained in the cDNA library clone, we were able to PCR amplify a full-length cDNA from K14B06 TCC. The open reading frame in this cDNA codes for a protein that we called NKG2H. Alignment of NKG2H peptide sequence to other members of the NKG2 family is shown in Fig. 5. Comparison of NKG2H cDNA nucleotide sequence with NKG2 genomic sequences revealed that NKG2H is, in fact, an alternative spliced form of NKG2E in which intron VI has not been removed and that does not contain exon VII. In this case, the splice donor site of exon VI in NKG2E gene is not utilized. Therefore, the end of the open reading frame and the following 3′ untranslated sequences are identical to exon VI and the beginning of the intron VI of NKG2E gene (31).

It has been recently reported that the CD94/NKG2C activatory receptor is stabilized in the cell membrane and exerts its activatory function by means of the association with the transducing polypeptide DAP-12 (32), which takes place through a charged lysine residue in the transmembrane domain of NKG2C. As NKG2H also possesses a charged lysine residue in its transmembrane portion, we tested whether it can be associated to CD94 and DAP-12 in the cell membrane. To that purpose, we constructed a plasmid encoding a GFP-NKG2H chimera protein and cotransfected it together with CD94 and DAP-12 cDNAs into COS-7 cells. To assess whether we could detect green fluorescence on the cellular membrane, we performed double fluorescence experiments. While in cells transfected with pEGFP-NKG2H alone, the protein was retained in the endoplasmic reticulum, and no green fluorescence was detected on the cell surface (Fig. 6, *D* and *F*). In cells cotransfected with the three cDNAs, the GFP-NKG2H chimera protein was localized to the cellular membrane (Fig. 6*A*). This was corroborated by staining of the cellular surface with an anti-CD94 mAb and a red fluorochrome (Fig. 6, *B* and *C*). Similar results were

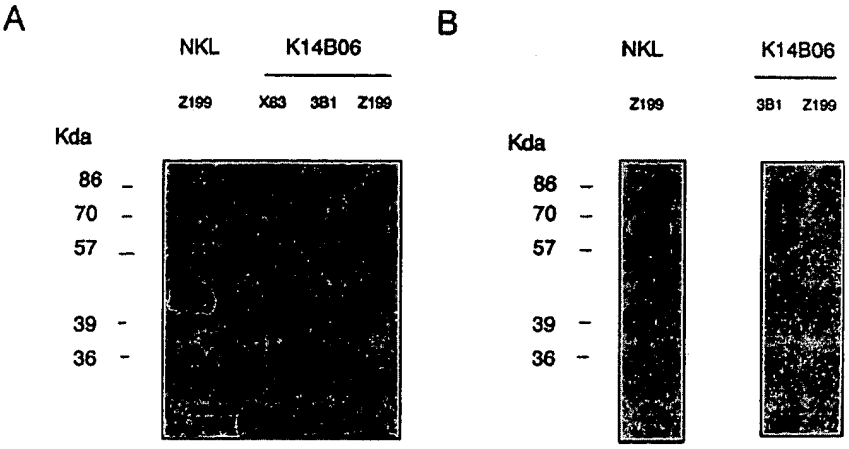



**FIGURE 4.** K14B06 activating heterodimer is composed of CD94, and a 39-kDa polypeptide. NKL cell line or K14B06 TCC were surface labeled with $^{125}I$, lysed, and proteins were immunoprecipitated with either Z199 mAb (anti-CD94/NKG2A), 3B1 MAb (anti-CD94), or control P3X63 myeloma supernatant. Samples were analyzed in 10% SDS-PAGE under reducing (*A*) or nonreducing conditions (*B*).

obtained upon transfection with a plasmid encoding a FLAG-NKG2H-tagged protein. Anti-FLAG M2 mAb was able to stain the surface of NKG2H-FLAG plus CD94-transfected cells only upon cotransfection of a DAP-12 expression plasmid (data not shown).

## Discussion

This paper shows the TCR-independent activation of an $\alpha\beta$ TCC triggered by the cross-linking of a CD94/kp39 heterodimer. The engagement of CD94 heterodimers expressed on K14B06 results in early activation events, such as $Ca^{2+}$ mobilization, and in late effector functions, such as induction of cytolytic activity and IFN-$\gamma$ production. These results are unexpected and differ from those described with other T cell costimulatory/accessory molecules, including activatory NK receptors, such as p50 molecules (KIR2DS) (14, 16, 17) or human NKR-PA1, another type II membrane glycoprotein of the C-type lectin superfamily (33). In these reports, KIR2DS proteins are trigger receptors for IFN-$\gamma$ secretion but only acted as coactivatory receptors with regard to T cell proliferation or killing, being capable of altering the strength of T cell response at a given Ag dose, but never inducing cytolytic activity. None of the other membrane molecules studied was able to activate the K14B06 TCC in a TCR-independent manner. Therefore, the cytotoxicity triggered by the aggregation of CD94/kp39 heterodimer on K14B06 TCC is a property that does not seem to be shared by other costimulatory molecules expressed on T lymphocytes.

The aggregation of the CD94/NKG2 dimers only activated redirected lysis or up-regulated CD25 Ag in one of the eight $CD94^+/Z199^-$ TCC assayed, suggesting a high heterogeneity either in the molecular structure of the CD94 heterodimer or in the transduction pathway among T cells. Since we were able to identify DAP-12 expression by RT-PCR in K14B06, but not in two other $CD94^+NKG2A^-$ TCC, the absence of the associated DAP-12 signal transducing molecule could at least explain the functional defect upon CD94 engagement in some $CD94^+NKG2A^-$ TCC (data not shown). However, it is also possible that other mechanisms could account for this functional heterogeneity. We also observed that K14B06 TCC had the highest membrane expression of CD94 by flow cytometry analysis among all of the $CD94^+NKG2A^-$ TCC tested (data not shown). We were unable to unequivocally identify the activatory CD94 heterodimer expressed on K14B06 TCC. The immunoprecipitation assays showed a 39-kDa glycoprotein very similar to that found in NK cells with CD94 activatory phenotype (28). The expression of mRNA encoding NKG2H, an

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NKG2A   MDNQGVIYSDLNLPPNPKRQQRKPKGNKSSILATEQEITYAELNLQKASQDFQGNDKTYHCKDLPSAP   68
NKG2C   MSKQRGTFSEVSLAQDPKRQQRKPKGNKSSISGTEQEIFQVELNLQNPSLNHQGIDKIYDCQGLLPPP   68
NKG2E   MNKQRGTFSEVSLAQDPKPQQRKPKGNKSSISGTEQEIFQVELNLQNASLNHQGIDKIYDCQGLLPPP   68
NKG2H   MNKQRGTFSEVSLAQDPKPQQRKPKGNKSSISGTEQEIFQVELNLQNASLNHQGIDKIYDCQGLLPPP   68

NKG2A   EKLIVGILGIICLILMASVVTIVVIPSTLIQRHNNSSLNTRTQKARHCGHCPEEWITYSNSCYYIGKE  136
NKG2C   EKLTAEVLGIICIVLMATVLKTIVLIPFLEQNNSSP--NTRTQKARHCGHCPEEWITYSNSCYYIGKE  134
NKG2E   EKLTAEVLGIICIVLMATVLKTIVLIPFLEQNNSSP--NTRTQKARPCGHCPEEWITYSNSCYYIGKE  134
NKG2H   EKLTAEVLGIICIVLMATVLKTIVLIPFLEQNNSSP--NTRTQKARPCGHCPEEWITYSNSCYYIGKE  134

NKG2A   RRTWEESLLACTSKNSS-LLSIDNEEEMKFLSIISPSSWIGVFRNSSHHPWVTMNGLAFKHEIKDSDN  203
NKG2C   RRTWEESLLACTSKNSS-LLSIDNEEEIKFLASILPSSWIGVFRNSSHHPWVTINGLAFKHKIKDSDN  201
NKG2E   RRTWEESLQACASKNSSSLLSIDNEEEMKFLASILPSSWIGVFRNSSHHPWVTINGLAFKHEIKDSDH  202
NKG2H   RRTWEESLQACASKNSSSLLSIDNEEEMKFLASILPSSWIGVFRNSSHHPWVTINGLAFKHEIKDSDH  202

NKG2A   AELNCAVLQVNRLKSAQCGSSII----------YHCKHK-----L                         233
NKG2C   AELNCAVLQVNRLKSAQCGSSMI----------YHCKHK-----L                         231
NKG2E   AERNCAMLHVRGLISDQCGSSRIIRRGFIMLTRL----------VLNS                       240
NKG2H   AERNCAMLHVRGLISDQCGSSRIIVSISFRIKALELAVHQIKFYICSNRNDIMIA                 257
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**FIGURE 5.** Alignment of NKG2 family members. The alignment was generated by the Clustal method. Gaps (dashes) were introduced to maximize homologies. Dots indicate amino acid identity, and bars indicate conservative changes. ITIM motifs within NKG2A sequence and transmembrane segments are underlined. Conserved cysteines are highlighted. Amino acids are numbered on the right side. Nucleotide sequence for NKG2H cDNA is available from GenBank with accession no. AF078550.

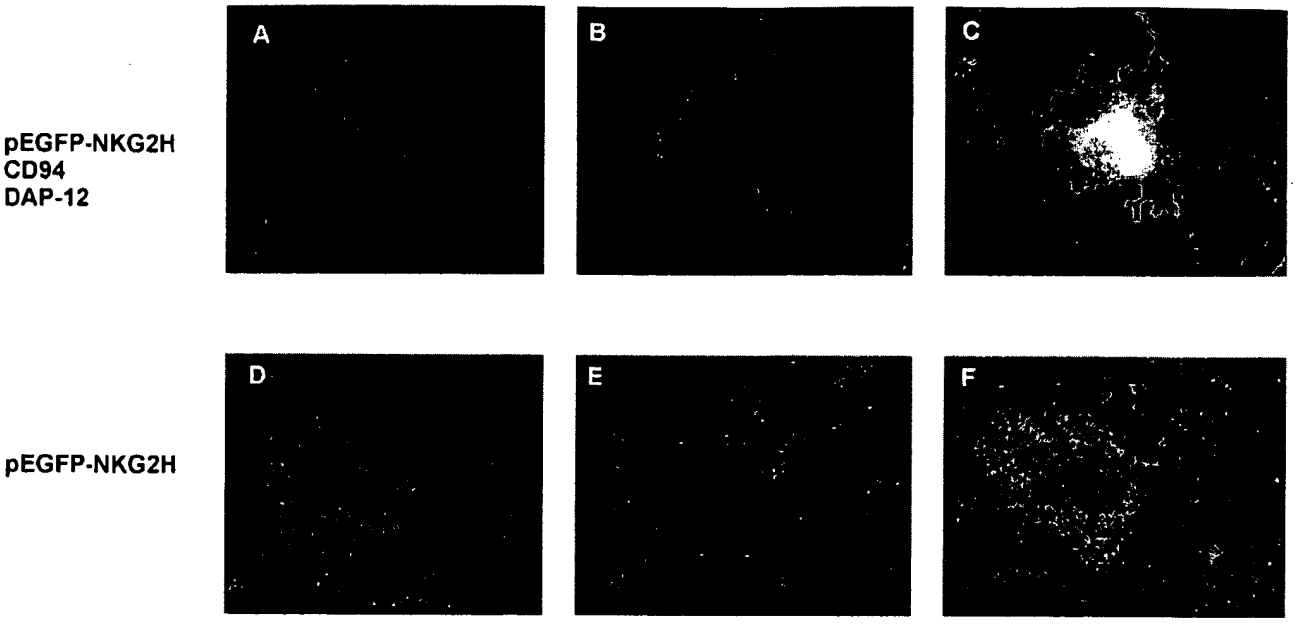


**FIGURE 6.** NKG2H molecule is transported to the cellular membrane when coexpressed with CD94 and DAP-12. COS-7 cells were either transfected with pEGFP-NKG2H plasmid (*D*, *E*, and *F*) or cotransfected with pEGFP-NKG2H, pJFE14-CD94, and pJFE14-DAP-12 expression plasmids (*A*, *B*, and *C*). Forty-eight-hour posttransfection cells were fixed and stained with 3B1 (anti-CD94) mAb followed by Cy-3 goat anti-mouse (GAM) IgG (*upper panels*) or with TS2/16 (anti-VLA-$\beta$1) mAb and Cy-3 GAM IgG (*lower panels*). GFP-NKG2H fluorescence was visualized in *A* and *D*. Red fluorescence staining of CD94 is shown in *B*, and red fluorescence staining of VLA-$\beta$1 is shown in *E*. *C* and *F*, Both green and red fluorescence were photographed on the same frame by double exposure.

alternative spliced form of NKG2E gene that lacks the ITIMs found on NKG2A and killer inhibitory receptors (KIR) and contains a charged residue in its transmembrane domain, leads us to hypothesize that this molecule could be part of the activating CD94 heterodimer expressed on K14B06 T cells. The cotransfection experiments shown in Fig. 6, together with the 39-kDa band immunoprecipitated in COS cells also cotransfected with plasmids coding for CD94, DAP-12, and the FLAG-NKG2H-tagged protein (data not shown), support this hypothesis. Accordingly, the sequence of several PCR products obtained from K14B06 TCC with the primers used to amplify NKG2C, always yielded NKG2H sequence. This result suggests that NKG2H mRNA should be more abundant than NKG2C mRNA. However, we have not formally excluded that NKG2C could also be present on K14B06 cell membrane and could be responsible for the CD94-triggered T cell activation. The fact that HLA-E molecule loaded with HLA-G1 peptide is not recognized by the CD94/kp39 heterodimer expressed on the K14B06 TCC (although it triggers the lysis of RMA-S cells by two NK clones expressing the CD94/NKG2C heterodimer), suggests that CD94/NKG2C heterodimer is either not expressed or not fully functional upon HLA-E recognition in this TCC.

It is possible that the activatory CD94/NKG2H heterodimer expressed on K14B06 TCC could be part of the innate recognition system, recognizing nonself structures expressed by transformed or infected cells that do not need to be related to MHC molecules. The secretion of IFN-$\gamma$, a cytokine strongly associated with antimicrobial immunity, also suggests that activatory CD94 heterodimer(s) present on K14B06 could react with molecular patterns present on different microorganisms or infected cells rather than with individual structures (34). However, the structural similarity between NKG2H and other members of the NKG2 family opens the possibility that the ligand specificity of CD94/kp39 on K14B06 was related to that of the CD94/NKG2A or CD94/NKG2C heterodimers (HLA-E molecules) (11, 14). To preserve self-tolerance, the activatory CD94 heterodimer should interact either with nonself MHC-like molecules, such as those described on some viruses, or with MHC molecules loaded with peptides that are either shared by some pathogens or that changed the conformation of the class I molecule in an identical way (35–38). Alternatively, the activatory CD94 heterodimer could recognize self-MHC molecules that have incorporated self peptides. However, the TCR-independent cytotoxicity triggered by the CD94/kp39 heterodimer on K14B06 TCC should require powerful control mechanisms to avoid autoreactivity. The analysis of NK cells has shown that the presence of stimulatory antiself receptors does not lead to lysis of autologous cells if the inhibitory signals transduced by the KIRs are the dominant controllers of the cytotoxic activity (1–4). Although K14B06 TCC express Ig-like transcript 2 (ILT2), the addition of a mAb specific for this molecule (HP-F1) (24) was not able to inhibit CD94-redirected lysis (data not shown). Nevertheless, other still undefined membrane molecules could balance the CD94/kp39-dependent cytotoxicity. Interestingly, the CD94-triggered lysis, but not the CD3-mediated redirected cytotoxicity, was specifically inhibited by anti-CD48 (data not shown), suggesting that CD94/NKG2-derived activatory signals are not identical to those triggered by TCR/CD3 engagement and can be specifically regulated.

In summary, we have described for the first time the triggering of cytotoxicity upon CD94/kp39 heterodimer engagement on cytolytic $\alpha\beta$ T cells and the sequence of NKG2H, a novel NKG2 family protein able to associate to CD94. The activatory CD94/NKG2 heterodimer present on a subset of $CD8^+$ T cells could be involved in the innate immunity and may function as a source of the proinflammatory cytokine IFN-$\gamma$.

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